

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
F. Charles Brunicardi

Application No.: 10/656,450 (publ'd as US20050059620) Confirmation No.: 8472

Filed: Sept. 5, 2003

Art Unit: 1632

For: PROMOTER DRIVEN TISSUE SPECIFIC
CYTOTOXIC AGENTS AND METHODS OF USE

Examiner: Magdalene K. Sgagias

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION MAILED 03/03/2006

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Office Action dated March 3, 2006, please amend the above-identified U.S. patent application as indicated below.

Amendments to the Specification are provided on pages 2 to 3 of this paper.

Amendments to the Claims are reflected in the listing of claims on pages 4 to 7 of this paper.

Remarks begin on page of this paper 8.

AMENDMENTS TO THE SPECIFICATION

The title for this pending Application Serial No. 10/646,450 is:

**PROMOTER DRIVEN TISSUE SPECIFIC CYTOTOXIC AGENTS AND
METHODS OF USE**

Please replace the paragraph under PRIOR RELATED APPLICATIONS on page 1 of the specification [i.e., please replace paragraph 0001 of US20050059620] with the following replacement paragraph:

This application claims the benefit of U.S. application Ser. No. 09/686,631 filed on Oct. 11, 2000 and issued as US Pat. No. 6,716,824 on Apr. 6, 2004. It also claims the benefit of U.S. Provisional Patent Application No. 60/161,109, filed Oct. 22, 1999, and U.S. Provisional Patent Application No. 60/224,382, filed Aug. 9, 2000.

A clean version of this replacement paragraph is as follows:

This application claims the benefit of U.S. application Ser. No. 09/686,631 filed on Oct. 11, 2000 and issued as US Pat. No. 6,716,824 on Apr. 6, 2004. It also claims the benefit of U.S. Provisional Patent Application No. 60/161,109, filed Oct. 22, 1999, and U.S. Provisional Patent Application No. 60/224,382, filed Aug. 9, 2000.

AMENDMENTS TO THE CLAIMS

1 – 112. (previously canceled)

113 – 118. (canceled without prejudice)

119. (previously presented) A method of killing a pancreatic tumor cell in a subject, the method comprising:

- a) administering to a subject a nucleic acid comprising a vector with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a pancreatic tumor cell that does not express insulin,
- b) administering a prodrug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the pancreatic tumor cell that does not express insulin.

120. (previously presented) The method of claim 119, where the cytotoxic gene is the thymidine kinase gene.

121. (previously presented) The method of claim 119, where the cytotoxic gene is the thymidine kinase gene and the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside.

122. (previously presented) The method of claim 121, wherein the administration is systemic.

123. (previously presented) The method of claim 121, wherein the administration is by direct administration at the site of the pancreatic tumor cell.

124. (previously presented) A method of treating pancreatic tumor cells in a subject, the method comprising:

- a) administering to a subject a nucleic acid comprising a vector with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a PDX-1 positive pancreatic tumor cell,
- b) administering a prodrug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the PDX-1 positive pancreatic tumor cell.

125. (currently amended) The method of claim ~~123~~ 124, where the cytotoxic gene is the thymidine kinase gene.

126. (currently amended) The method of claim ~~123~~ 124, where the cytotoxic gene is the thymidine kinase gene and the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside.

127. (previously presented) A method of killing a pancreatic tumor cell in a subject, the method comprising:

- a) administering to a subject a nucleic acid comprising a vector with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a pancreatic tumor cell,
- b) administering a prodrug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the pancreatic tumor cell.

128. (previously presented) The method of claim 127, where the cytotoxic gene is the thymidine kinase gene.

129. (previously presented) The method of claim 127, where the cytotoxic gene is the thymidine kinase gene and the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside.

130. (previously presented) The method of claim 129, wherein the administration is systemic.

131. (previously presented) The method of claim 129, wherein the administration is by direct administration at the site of the pancreatic tumor cell.

132. (previously presented) A method of killing a tumor cell in a subject, the method comprising:

- a) administering to a subject with a tumor cell expressing PDX-1, a nucleic acid comprising an adenoviral vector with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in the tumor cell expressing PDX-1,
- b) administering a pro-drug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the tumor cell expressing PDX-1.

133. (previously presented) The method of claim 132, where the cytotoxic gene is the thymidine kinase gene.

134. (previously presented) The method of claim 132, where the cytotoxic gene is the thymidine kinase gene and the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside.

135. (previously presented) The method of claim 132, wherein the administration is systemic.

136. (previously presented) A method of killing a tumor cell in a subject, the method comprising:

- a) administering to a subject with a tumor cell expressing PDX-1 a nucleic acid comprising a vector with an insulin promoter, said insulin promoter comprising multiple copies of SEQ ID NO: 2 operatively coupled to multiple copies of SEQ ID NO: 3 or 4, said insulin promoter operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in the tumor cell expressing PDX-1,
- b) administering a pro-drug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the tumor cell expressing PDX-1.

137. (previously presented) The method of claim 136, where the cytotoxic gene is the thymidine kinase gene.

138. (previously presented) The method of claim 136, where the cytotoxic gene is the thymidine kinase gene and the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside.

139. (previously presented) The method of claim 136, wherein the administration is systemic.

REMARKS

In view of the above amendments and the following remarks, Applicant believes that all stated objections and rejections of the Office Action mailed March 3, 2006 are overcome, and Applicant respectfully requests that all pending claims be allowed to pass to issuance.

Re: Title

The Filing Receipt mailed January 30, 2004 for this pending Application Serial No. 10/656,450 incorrectly listed the application's title as "Promoter driven tissue specific cytotoxic agents and **title** methods of use" (emphasis added). In response to a Request by Applicant mailed February 9, 2005, the USPTO issued a Corrected Filing Receipt that (correctly) listed the application's title as "Promoter driven tissue specific cytotoxic agents and methods of use" (a subsequently received Notice of Publication also correctly listed the application's title as "Promoter driven tissue specific cytotoxic agents and methods of use"). However, when the application published on March 17, 2005, as US20050059620, it published with the incorrect title "PROMOTER DRIVEN TISSUE SPECIFIC CYTOTOXIC AGENTS AND **TITLE** METHODS OF USE" (emphasis added). Accordingly, Applicant reiterates that the application's title is "PROMOTER DRIVEN TISSUE SPECIFIC CYTOTOXIC AGENTS AND METHODS OF USE." Applicant's inclusion of this correct title in the "Amendments to the Specification" is provisional in nature (i.e., done only if the application's correct title is not yet listed in files, particularly electronic files, of the USPTO).

Re: Informalities in the Specification and Claims

The Office Action (page 2) states an objection to the disclosure as follows: "Applicant fail[s] to incorporate in line 2 [i.e., under prior related applications] the issue date of the patent number 6716824."

In response, Applicant has amended the first sentence of the paragraph of the specification under the heading PRIOR RELATED APPLICATIONS to read: "This application claims the benefit of U.S. application Ser. No. 09/686,631 filed on Oct. 11, 2000 and issued as US Pat. No. 6,716,824 on Apr. 6, 2004."

The Office Action (page 2) notes that “[c]laims 113–118 are not entered” and states a requirement that a corrected list of all pending claims be provided.

In response, Applicant respectfully notes that, in listing claims 113–118 above, Applicant lists these claims without claim text and without using the claim identifier “not entered” (Applicant used the claim identifier “not entered” in both the Preliminary Amendment mailed Sept. 5, 2003 and the Second Preliminary Amendment mailed Feb. 26, 2004). Applicant instead uses the claim identifier “canceled without prejudice” (which the USPTO has stated is another acceptable alternative -- see <http://www.uspto.gov/go/og/2005/week27/patamnd.htm>). Applicant provides the above list of all pending claims as a correct list.

The Office Action (page 2) states objections to claims 125 and 126 as being duplicates of claims 120 and 121.

In response, Applicant respectfully traverses these objections for amended claims 125 and 126 in that amended claims 125 and 126 each depend from claim 124 (not claim 123), and claims 120 and 121 each depend from claim 119. Although claims 125 and 126 have dependent elements in common with claims 120 and 121, respectively, claims 125 and 126 are not duplicates of claims 120 and 121 because independent claims 124 and 119 (i.e., the independent claims from which the dependent claim pairs respectively depend) are not duplicates.

The Office Action (page 2) states objections to claims 119, 124, 127, 132 and 136 “because the phrase ‘operatively coupled to’ is not a recognized art term for a construct composition.”

In response, Applicant respectfully traverses these objections and notes the following. First, Applicant uses the phrase “operatively linked to” (e.g., see each of paragraphs 0011–0020, 0078, 0091, 0094, 0097, 0099, 0100 and 0102 of corresponding published application US20050059620) in an interchangeable manner with “operatively coupled to” (e.g., see “operatively linked or coupled” of paragraph 0088; see also the Applicant’s use of the phrase “coupled to” in paragraphs 0039, 0040, 0042, 0045 and 0088). As a result, a person of ordinary skill would have no doubt about the meaning of the phrase “operatively coupled to.” In addition, Applicant maintains that the phrase “operatively coupled to” is a recognized art term for a construct composition. For example, “operatively coupled to” is an element of each of the three claims of priority parent application 09/686,631 (now U.S. Pat. No. 6,716,824 “Treatment of

pancreatic adenocarcinoma by cytotoxic gene therapy”), as well as of claims of the following recombinant nucleic acid molecule-related patents: U.S. Pat. Nos. 5,807,707 “High efficiency translation of mRNA molecules” (claims 20–37), 5,827,654 “Basal body rod protein genes of campylobacter” (claims 4–9), 6,140,082 “Expression of gene products from genetically manipulated strains of Bordetella” (claims 1–31), and 6,358,727 “Haemophilus transferrin receptor genes” (claims 5–9), among many others.

In view of the above, Applicant respectfully requests that all objections to the specification and pending claims for the stated informalities be withdrawn, and that the application be allowed to pass to issuance.

Re: Double Patenting

The Office Action (pages 2–3) states nonstatutory double patenting rejections of claims 119–121, 123–129, 131–134, 136–139 over claims 1–3 of U.S. Pat. No. 6,716,824.

In response, Applicant respectfully notes that the patent terms of both a patent issuing from the pending application and U.S. Pat. No. 6,716,824 will very likely be identical (barring any required patent term adjustments for the present application) under the twenty-years-from-filing provisions of 35 U.S.C. § 154. Applicant respectfully requests that the issue of double patenting (e.g., either a further responsive argument, if needed, or a terminal disclaimer) be held in abeyance until claims in the pending application are allowed.

Re: Written Description Requirement

The Office Action (pages 3–5) states rejections of claims 119, 124, 127, 132 and 136 as failing to meet the written description requirement under 25 U.S.C. § 112.

In response, Applicant respectfully traverses these rejections. While the Office Action (page 4) acknowledges that “[t]he specification describes that the present invention is directed to an RIP-tk (rat insulin promoter thymidine kinase) construct that targets pancreatic cells,” the Office Action (page 4) further asserts “the specification does not provide any disclosure as to what would have been the complete structure of a representative number of cytotoxic genes either within one species or among different species necessary to target

pancreatic tumor cells in a subject.” Applicant respectfully disagrees. For example, the specification (page 18, paragraph 86; i.e., paragraph 0086 of US20050059620) provides:

[86] The present invention is not limited to thymidine kinase as the cytotoxic gene. As will be clear to those of skill in the art, various cytotoxic genes may be used. For example, the cytotoxic gene may be a directly cytotoxic gene, such as the gene encoding diphtheria toxin, the gene encoding ricin or the gene encoding caspase. Caspase is a gene product that promotes cell death by apoptosis. Alternately, the cytotoxic gene may be a suicide gene, such as the aforementioned gene encoding thymidine kinase. Suicide genes can make targeted cells susceptible to specific drugs. Administering the drug to cells carrying such suicide genes results in cell death. For example, cells expressing the thymidine gene are killed following treatment with GCV or a similar drug, whereas cells not expressing the thymidine kinase gene are unharmed by GCV treatment.

Consequently, in addition to describing thymidine kinase as a member of the genus of cytotoxic genes, the application describes at least three other members of this genus: (1) the gene encoding diphtheria toxin, (2) the gene encoding ricin and (3) the gene encoding caspase.

The specification also describes constructs for targeting other tissues. For example, the specification (page 20, paragraph 93; i.e., paragraph 0093 of US20050059620) provides:

[93] The present invention is not limited to the RIP-tk construct disclosed herein. The present invention is also broadly directed to methods and constructs for targeting other tissues using unique promoters or genes specific to a particular tissue, to deliver a cytotoxic or suicide gene, such as tk, to the tissue to then kill the tumor. For example, in one embodiment of the invention, breast tissue may be targeted by linking a suicide gene or other cytotoxin with an estrogen receptor promoter and/or a casein promoter. In another embodiment, liver tissue may be targeted by using the a fetal protein promoter.

Consequently, in addition to describing a cytotoxic or suicide gene linked to rat insulin promoter (RIP) for targeting pancreas tissue, the application describes a cytotoxic or suicide gene linked to at least three other tissue-targeting promoters: (1) an estrogen receptor promoter (for targeting breast tissue), (2) a casein promoter (also for targeting breast tissue) and (3) a fetal protein promoter (for targeting liver tissue).

With regard to describing species for targeting pancreatic tumor cells in a subject, the application describes a multitude of promoter molecules. For example, the specification (page 21, paragraph 95; i.e., paragraph 0093 of US20050059620) provides:

[95] The insulin promoter may generally be any insulin promoter, preferably is a rat insulin promoter, and more preferably is SEQ ID NO:1. Alternatively, the insulin promoter can be an effective fragment of SEQ ID NO:1. An effective fragment is a truncation of SEQ ID NO:1 which exhibits substantially the same transcription ability as does SEQ ID NO:1. The effective fragment preferably exhibits at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, and ideally 100% of the transcription ability of SEQ ID NO:1. The effective fragment is preferably at least about 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of the length of SEQ ID NO:1. Alternatively, the insulin promoter can be a promoter having a high level of percent sequence identity to SEQ ID NO:1. The level of percent sequence identity is preferably at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity as compared to SEQ ID NO:1. Percent sequence identity is determined by aligning the two sequences with a commercial software package such as CLUSTALW version 1.6 (Thompson, J. D., et al. *Nucleic Acids Res.* 22(22):4673-4680 (1994)). The number of matches between the two aligned sequences is divided by 502 and multiplied by 100 in order to obtain a percent sequence identity.

In describing use of an algorithm for determining percent sequence identity (as in paragraph 95), as well as for use in distinguishing classes of promoter molecules that are related to the insulin promoter molecule of SEQ ID NO: 1 (and fragments thereof), the specification provides additional written description support for a multitude of promoter molecules.

The specification also describes insulin promoter molecules that further comprise a nucleic acid sequence encoding one or more transcription factors. For example, the specification (page 23, sixth sentence of the paragraph above the paragraph numbered "[98]" i.e., the sixth sentence of paragraph 0098 of US20050059620) provides: "The molecule may further comprise a structural nucleic acid sequence encoding one or more transcription factors selected from the group consisting of the BETA-2 transcription factor, the GATA4 transcription factor, the E47 transcription factor, and the PDX-1 transcription factor."

In view of the above, Applicant respectfully requests that rejections to pending claims for 119, 124, 127, 132 and 136 for failing to meet the written description requirement be withdrawn, and that the claims be allowed to pass to issuance.

Re: Enablement Requirement

The Office Action (pages 5-13) states rejections of claims 119-139 as failing to meet the enablement requirement under 25 U.S.C. § 112.

In response, Applicant respectfully traverses these rejections. First, Applicant notes that the Office Action (pages 5 and 8) organizes its rejections by the following elements within the following claim groups (underlining is simply for pedagogical or heuristic purposes):

Claim Group	Assertion (page 8): <i>"specification fails to provide any relevant teachings or specific guidance and/or working examples with regard to:"</i>
a) 119-123	<i>"killing of a <u>pancreatic tumor cell</u> that does not express insulin in a subject by administering a <u>construct</u> with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug"</i>
related claim elements	method of <u>killing</u> a pancreatic tumor cell in a subject. . . administering to a subject a <u>nucleic acid comprising a vector</u> with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a <u>pancreatic tumor cell that does not express insulin</u> . . .
b) 124-126	<i>"treating a <u>PDX-1 positive pancreatic tumor cell</u> in a subject by administering a <u>construct</u> with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug"</i>
related claim elements	method of <u>treating</u> pancreatic tumor cells in a subject . . . administering to a subject a <u>nucleic acid comprising a vector</u> with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a <u>PDX-1 positive pancreatic tumor cell</u>
c) 127-131	<i>"killing a <u>pancreatic tumor cell</u> in a subject by administering a <u>construct</u> with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug"</i>
related claim elements	method of <u>killing</u> a pancreatic tumor cell in a subject. . . administering to a subject a <u>nucleic acid comprising a vector</u> with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a <u>pancreatic tumor cell</u>
d) 132-135	<i>"killing a <u>PDX-1 positive tumor cell</u> in a subject by administering an <u>adenoviral vector</u> with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug"</i>
related claim elements	method of <u>killing</u> a tumor cell . . . administering to a subject with a tumor cell expressing PDX-1, a <u>nucleic acid comprising an adenoviral vector</u> with an insulin promoter having SEQ ID NO:1 operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in the <u>tumor cell expressing PDX-1</u>
e) 136-139	<i>"killing a <u>PDX-1 positive tumor cell</u> in a subject by administering to a subject a vector with an insulin promoter having SEQ ID NO:2 operatively linked to multiple copies of SEQ ID NO:3 or 4, said insulin promoter operatively linked to a cytotoxic gene and a prodrug"</i>
related claim elements	method of <u>killing</u> a tumor cell . . . administering to a subject with a tumor cell expressing PDX-1 a <u>nucleic acid comprising a vector</u> with an insulin promoter, said insulin promoter comprising multiple copies of SEQ ID NO:2 operatively coupled to multiple copies of SEQ ID NO:3 or 4, said insulin promoter operatively coupled to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in the <u>tumor cell expressing PDX-1</u>

For the convenience of review, Applicant addresses the enablement rejections according to this same general organization.

Killing of a pancreatic tumor cell that does not express insulin

The Abstract discloses that pancreatic adenocarcinoma (PDA) cells are pancreatic tumor cells that typically do not express insulin, and Example 2 describes “Treatment of human ductal pancreatic adenocarcinoma tumors *in vivo*” (see paragraphs 168–172, i.e., paragraphs 0170–0174 of US20050059620). In particular, *in vivo* delivery of RIP-tk in combination with GCV resulted in a significant decrease in tumor burden in SCID mice; the combination killed all PANC-1 tumors in eight of nine mice ($p < 0.05$ compared to all other groups, ANOVA) (paragraph 171). PANC-1 cells are human pancreatic ductal adenocarcinoma cells (see paragraph 42). In enabling the killing of PANC-1 cells *in vivo*, the specification enables the killing of a pancreatic tumor cell in a subject where the pancreatic tumor cell does not express insulin.

Treating a PDX-1 positive pancreatic tumor cell

First, as defined at paragraph 53 (i.e., paragraph 0053 of US20050059620), “‘PDX-1 positive’ means cells that naturally contain or comprise, or are modified to contain or comprise, a PDX-1 transcription factor.” That same paragraph begins by noting that PDX-1 positive human pancreatic ductal carcinoma cells can be targeted using a RIP-tk construct.

Further enabling description for treating a PDX-1 positive pancreatic tumor cell is provided (as introduced in paragraph 57) in Examples 2–4 (i.e., paragraphs 134–181, i.e., paragraphs 0137–183 of US20050059620). Example 2 initially describes *in vitro* studies in which a significant increase in cell death was observed in PDX-1 positive PANC-1 and CAPAN-1 cells transfected with RIP-tk while no significant increase in cell death was observed in PDX-1 negative MIA-1 cells transfected with RIP-tk (paragraphs 134–135, i.e., paragraphs 0138–0139 of US20050059620). Example 2 then describes, as noted above, *in vivo* delivery of RIP-tk in combination with GCV in SCID mice. This treatment resulted in a significant decrease in tumor burden and killed all PANC-1 tumors in eight of nine mice ($p < 0.05$ compared to all other groups, ANOVA) (paragraph 171). In enabling the treatment of PANC-1 cells *in vivo*, the specification enables the treatment of a PDX-1 positive pancreatic tumor cell in a subject.

Killing a pancreatic tumor cell

The enabling description for killing a pancreatic tumor cell that does not express insulin and for treating a PDX-1 positive pancreatic tumor cell (as detailed above) also provides enabling description for killing a pancreatic tumor cell in a subject. In addition to providing description for killing pancreatic adenocarcinoma (PDA) tumor cells, the specification provides in various places (e.g., paragraph 92) description for other pancreatic tumor cell types that may be targeted for ablation (e.g., β -cell tumor cells and insulinoma cells).

Killing a PDX-1 positive tumor cell using an adenoviral vector delivery

The specification details at paragraph 79 that an agent [containing a recombinant nucleic acid sequence] "is delivered by infecting with a recombinant viral vector, such as a recombinant adenovirus." The specification further details at paragraph 79 that "a gutless adenovirus may be prepared per the protocol of Hardy et al. (Smyth-Templeton N, et al., 15 NATURE BIOTECHNOL. 647-652 (1997); Hardy S, et al., 71(3) J. VIROL. 1842-1849 (1997)), or by using the gutless adenovirus available from the Shell Center for Gene Therapy, Baylor College of Medicine (Houston, Tex.)."

Within the field of gene therapy, adenoviral vector delivery of cytotoxic genes may be considered to have been reasonably well developed as of the filing date of the pending application. When the above-noted description (including citation to the protocol of Hardy et al.) is viewed in the context of the specification's description of pioneering work in targeting and killing PDX-1 positive tumor cells using a RIP-tk construct, the specification enables killing a PDX-1 positive tumor cell using adenoviral vector delivery of an insulin promoter-cytotoxic gene construct. A copy of the cited paper of Hardy et al. (1997) is enclosed in Appendix I of this Response.

Killing a PDX-1 positive tumor cell using a vector with an insulin promoter having SEQ ID NO:2 operatively linked to multiple copies of SEQ ID NOS:3 or 4

The specification describes the sequences of SEQ ID NOS: 2-4 in a table of sequence identifiers at paragraph 36 [. . . SEQ ID NO:2 is the BETA-2 site CANNTG. SEQ ID NO:3 is the PDX-1 site TAAT. SEQ ID NO:4 is the PDX-1 binding site CTTAAT . . .]. The specification provides the following additional description of these sequences at paragraph 46:

[46] It has been determined that 0.502 kb of RIP (SEQ ID NO:1) contains elements to maximally drive the expression of a gene. These elements include six BETA-2 sites (CANNTG; SEQ ID NO:2) and three PDX-1 sites (TAAT; SEQ ID NO:3), one of which contains the sequence CTTAAT (SEQ ID NO:4), which is the most favorable PDX-1 binding sequence. This smaller fragment allows for the development of smaller constructs. Such fragments make the creation of the constructs technically easier, increase the transfection efficiency *in vitro*, and aid in the development of *in vivo* gene delivery systems.

Within the field of gene therapy, basic technology for the production of recombinant nucleic acid molecules was very well developed as of the filing date of the pending application. In view of the above-noted disclosure, producing a vector with an insulin promoter having SEQ ID NO:2 operatively linked to multiple copies of SEQ ID NOS:3 or 4 could be readily accomplished without undue experimentation. Similarly, in view of the above-noted disclosure on killing a PDX-1 positive tumor cell, the specification enables killing a PDX-1 positive tumor cell using a vector with an insulin promoter having SEQ ID NO:2 operatively linked to multiple copies of SEQ ID NOS:3 or 4.

[remainder of page 16 intentionally left blank]

CONCLUSION

Applicant has addressed all issues of the Office Action of March 3, 2006, and Applicant again respectfully requests that the application be allowed to pass to issuance.

Applicant also notes a change of address for official correspondence (Applicant is also providing a separate form to facilitate this change of address). Applicant's previous representative, Dr. Tamsen Valoir, has moved from Applicant's firm, Jenkins & Gilchrist. Furthermore, Applicant's firm, Jenkins & Gilchrist, has moved to new offices. Please address future official communications for application serial no. 10/656,450 to:

Mark Gatschet, Ph.D. [of Customer No. **24238**]
Jenkins & Gilchrist, A Professional Corporation
5 Houston Center
1401 McKinney, Suite 2600
Houston, TX 77010

This response is being timely filed electronically **before** the expiration of the six-month deadline on Sept. 5, 2006. A fee for a three-month time extension from the three-month shortened statutory deadline of June 3, 2006 accompanies this Response {Sept. 3, the six-month date, falls on a Sunday, and Sept. 4 is Labor Day, a Federal holiday; accordingly, under 37 CFR §1.7 [Times for taking action; Expiration on Saturday, Sunday or Federal holiday], the deadline for filing a Response under a three-month time extension expires Sept. 5, 2006}.

Applicant's undersigned representative would very much appreciate an opportunity to discuss telephonically the pending claims with the Examiner (particularly before the Examiner were to consider mailing any communication other than a communication indicating allowance of claims). The direct dial number for Applicant's undersigned representative is 713-951-3309.

Respectfully submitted,

Dated: September 2, 2006

By Mark J. Gatschet
Mark J. Gatschet, Ph.D., Reg. No.: 42,569
JENKENS & GILCHRIST
5 Houston Center
1401 McKinney, Suite 2600
Houston, Texas 77010
(713) 951-3309 (phone); (713) 951-3314 (fax)
-- Attorneys for Applicant --

APPENDIX I

Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML (1997 Mar). "Construction of adenovirus vectors through Cre-lox recombination." *J Virol.* 71(3):1842-9.

Construction of Adenovirus Vectors through Cre-lox Recombination

STEPHEN HARDY,* MARINA KITAMURA, TONJA HARRIS-STANSIL, YUMIN DAI,
AND M. LISA PHIPPS

Somatix Therapy Corporation, Alameda, California 94501-1034

Received 9 September 1996/Accepted 15 November 1996

Two barriers prevent adenovirus-based vectors from having wide application. One is the difficulty of making new adenoviruses, and the second is the strong immunological reaction to viral proteins. Here we describe uses of Cre-lox recombination to overcome these problems. First, we demonstrate a simple method for constructing E1-substituted adenoviruses. Second, we demonstrate a method to construct adenovirus vectors carrying recombinant genes in place of all of the viral genes, so-called gutless adenovirus vectors. The pivotal feature in each method is the use of a negatively selected adenovirus named $\Psi 5$. We engineered a *cis*-acting selection into $\Psi 5$ by flanking its packaging site with *loxP* sites. When $\Psi 5$ was grown in cells making a high level of Cre recombinase, the packaging site was deleted by recombination and the yield of $\Psi 5$ was reduced to 5% of the wild-type level. To make a new E1-substituted virus, we used $\Psi 5$ as a donor virus and recombined it with a shuttle vector via a *loxP* site. The resulting recombinant virus has a single *loxP* site next to the packaging site and therefore outgrows $\Psi 5$ in the presence of Cre recombinase. To make a gutless virus, we used $\Psi 5$ as a helper virus. The only viral sequences included in the gutless vector are those needed in *cis* for its replication and packaging. We found that a *loxP* site next to the packaging site of the gutless virus was necessary to neutralize homologous recombination between $\Psi 5$ and the gutless viruses within their packaging domains.

Adenoviruses are attractive candidates for many gene delivery applications in medicine and science. Two particular features of adenovirus biology could be critical to successful use in gene therapy or molecular genetic experiments. First, the virus infects both resting and dividing cells of many types. Second, highly purified solutions of virus can easily be produced with titers of up to 10^{13} particles/ml. Additionally, decades of research have produced a relatively clear picture of the viral life cycle and most functions of the many viral proteins (reviewed in reference 24). Despite these attractive features, adenovirus has been only narrowly applied as a tool. A major barrier to general application of the virus is the process of making recombinant viruses. Generally, new adenoviruses are constructed by a process of overlap recombination followed by screening and plaque purification (3, 6, 20, 25). These steps are inefficient, technically demanding, and very time-consuming.

For most *in vivo* applications, there is a second serious limitation of adenoviruses. Adenoviruses are highly immunogenic, a property which has been maintained in current adenovirus vectors. The immunological reaction against adenoviruses can be thought of as having two phases, an initial reaction to the capsid proteins of the infecting virus and a later reaction against cells synthesizing viral proteins. Because of the complexity of adenovirus, most of the genome has been left intact in current vectors, and the remaining viral genes are able to direct protein synthesis, albeit at a reduced level. Therefore, cells transduced by these vectors are eliminated when viral genes in the vector begin to synthesize proteins (8, 28). Thus, these E1-deleted adenovirus vectors are powerful tools for experimentation in tissue culture systems and are restricted in their therapeutic uses to applications where only transient gene expression is needed or to those where a stimulation of immu-

nity is beneficial. The most practical route to overcome the immunological barrier to long-term gene transfer is to remove all of the viral genes from the vector, producing a "gutless" virus.

Keeping these difficulties in mind, we have developed methods for creating recombinant adenoviruses by using Cre recombinase from phage P1. Recombinant virus construction is driven by selection against a special adenovirus virus which we call $\Psi 5$. The negative selection is based on the observation that deletion of a sequence from 194 to 358 bp in the left end of adenovirus prevents the viral chromosome from being packaged into capsids (10, 11, 15). To make a conditional version of such a deletion, we flanked the packaging site in $\Psi 5$ by directly repeated *loxP* sites and constructed a 293 cell line called CRE8 which makes a high concentration of Cre recombinase (23). In these cells, recombination will delete the intervening packaging sequence in $\Psi 5$, producing an unpackable chromosome.

Using $\Psi 5$ and CRE8 cells, we have developed two methods to make either E1-substituted or gutless adenovirus vectors. To make an E1-substituted virus, we used $\Psi 5$ as a donor virus to supply the viral backbone. In addition to supplying negative selective pressure, Cre recombinase can catalyze recombination between $\Psi 5$ and a shuttle plasmid with a single *loxP* site, providing an efficient means to construct recombinants. Selective growth of the recombinant viruses was ensured by installing a normal packaging sequence on the shuttle plasmid. This combination of selection and recombination provided by Cre protein set up an arrangement to rapidly create new viruses.

In our second method, we used $\Psi 5$ as a helper virus to support replication and packaging of a gutless virus. Such a virus contains only those terminal sequences needed for replication and packaging of the viral chromosome surrounding nonviral DNA. The inverted terminal repeats (ITR) which serve as the origins of replications and a packaging sequence are two such sequences. Since the packaging site in the gutless virus does not have flanking *loxP* sites, the gutless virus should

* Corresponding author. Mailing address: Somatix Therapy Corporation, 850 Marina Village Pkwy., Alameda, CA 94501-1034. Phone: (510) 748-3029. Fax: (510) 769-8533. E-mail: shardy@apple.somatix.com.

be preferentially packaged. Growth of a mixed population of helper and gutless viruses on CRE8 cells should amplify the gutless virus.

MATERIALS AND METHODS

Cells and viruses. 293 and CRE8 cells were cultured in Dulbecco modified Eagle medium with 10% calf serum (12). The selective cell line CRE8 has a β -actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. The construction and properties of CRE8 cells are described elsewhere (13a). Viruses were used as freeze-thaw lysates, and all infections were done at a multiplicity of infection of 5 for each virus. Transfections were done according to Graham and van der Eb (13). Typically, a confluent 10-cm-diameter dish of 293 or CRE8 cells (1.6×10^7) was split into 16 6-cm-diameter dishes for transfection 2 to 4 h later. Each dish received 3 μ g of pAdlox or ploxAB plasmid and 3 μ g of 95 viral DNA in a final volume of 0.5 ml of CaPO₄, which was applied to the cells for 16 h. For the transfections using ploxAB shuttle plasmid, the medium was removed after 3 days and 1% Noble agar overlay in Dulbecco modified Eagle medium with calf serum was added. At 7 days, another layer of agar overlay containing 0.8 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per ml was added. Blue plaques were removed 1 day later.

For the experiment to determine the optimal way to deliver the 95 donor DNA, three plates of CRE8 cells were transfected with Ψ -digested pAdlox-B-gal DNA and infected with 95 virus at three different times, 4 h before transfection, at the same time as transfection, and at 18 h after transfection. Lysates were made 2 days after infection. The virus was then passed sequentially through CRE8 cells twice, and the packaged DNA was prepared from the second passage. For comparison, cells were cotransfected with 95 and pAdlox (gal DNAs). After 8 days, lysate from the cotransfection was used to infect CRE8 cells for packaged DNA. Finally, the DNA was digested with *Bsa*BI to distinguish between 95 and the recombinant DNAs.

Construction of vectors. Standard techniques were used to construct all plasmids. Plasmids were purified by the polyethylene glycol-LiCl technique (2). Frequently, rapid-prep DNA prepared by the alkaline lysis protocol was used for transfection to construct E1-substituted viruses (2). The success rate was similar to that for assays using purified plasmids (data not shown).

pCMV Ad, pAdlox, pAB, and derivatives. The shuttle plasmids for either E1-substituted or gutless virus construction were based on adenovirus type 5 (Ad5) and are derivatives of pCMV Ad. Each contains the following sequences in this order: (i) a left ITR, (ii) a packaging sequence, (iii) an expression cassette, and (iv) a right ITR (Fig. 1). Specifically, pCMV Ad begins with a *Sfi*I site, GGGCGGCTGGCGG, followed by nucleotides 2 to 553 from Ad5 (nucleotide 1 is the last C of the *Sfi*I site). This is connected to an *Xho*I site followed by the human cytomegalovirus immediate-early promoter from -600 to +1 relative to the start of transcription. The next segment is the polylinker of pSP73 from *Hind*III to *Eco*RI (Promega) and then polyadenylation signals from simian virus 40 (nucleotides 2752 to 2534 in the simian virus 40 genome). The poly(A) signals are followed by a pSP73 fragment from nucleotides 22 back through 2382 (containing *Clat*, *Eco*RV, and *Bgl*II sites) and an SP6 promoter). An *Apa*I site then connects this sequence to the right ITR, which ends with another *Sfi*I site. The *Sfi*I-bounded fragment is cloned between the *Pvu*II sites of pBluescript⁺, destroying the *Pvu*II sites (bases 533 back to 799) (Stratagene). Digestion of pCMV Ad with *Sfi*I cleaves the plasmid sequence away from the Ad5-based portion, allowing the Ad5 sequences to replicate in adenovirus-infected cells (data not shown) (14). To create pAdlox, a *Sfi*I site was inserted between the *Clat* and *Bgl*II sites in pCMV Ad, making pAdlox. The new sequence between *Clat* and *Bgl*II is ATGATGTCATCACTGATTAATGATATGCTATACGAAGTAT CACGATCT. (Note that the *Clat* site will be methylated if the plasmid is grown in *dam*⁺ *Escherichia coli*).

To monitor either overlap recombination or Cre-lox-mediated recombination, β -galactosidase reporter genes were inserted between the *Hind*III and *Sma*I sites of pAdlox or pCMV Ad. The *Bgl*II B fragment of Ad5 (3328 to 8914) was inserted into the unique *Bgl*II site in pCMV Ad, making pCMV Ad B with a β -galactosidase reporter gene. The shuttle plasmids were cut with *Sfi*I where indicated in Table 1.

The gutless shuttle plasmid pAB was constructed from pCMV Ad. The *Bgl*II A fragment from λ phage (nucleotides 22425 back to 415 in λ) is inserted between the *Bam*HI and *Bgl*II sites of pCMV Ad. Then a 4.9-kb β -galactosidase expression cassette was removed from pCMV Ad (a gift of J. Michael Bishop) by digestion with *Bam*HI and ligated into the *Bgl*II site, creating pAB. The size of the resulting ITR-bounded fragment is 28,350 bases. The gutless plasmid ploxAB was made from ploxAB (see below) and is identical to pAB, except that it has a single *loxP* site at position 193 relative to the left adenovirus origin.

95 and AdB-gal viruses. The 95 virus is an E1- and E3-deleted version of Ad5 containing *loxP* sites flanking the packaging site (Fig. 1). 95 was constructed by overlap recombination between a modified version of pAdlox and AdB-gal (8, 25). A second *loxP* site was inserted between nucleotides 193 and 194, the Ad5 left-end sequence in ploxAB. The orientations of the *loxP* sites are the same, and recombination between them will remove the packaging site. Then the *Bgl*II B fragment of Ad5 was inserted into the *Bgl*II site of ploxAB to

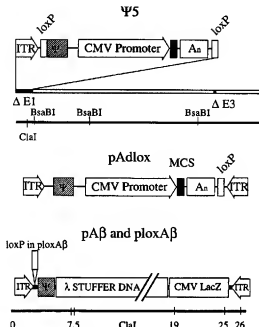


FIG. 1. Vector maps. Ψ , packaging site; CMV, cytomegalovirus; Aa, polyadenylation signal; MCS, multiple cloning site. (Top) A map of 95 virus. The *loxP* sites are directly repeated. The single *Clat* site is at 1475 between Aa and the right *loxP* site. The three *Bgl*II sites are at 2249, 9972, and 21620. (Middle) A map of the pAdlox shuttle plasmid for making E1-substituted adenoviruses. DNA sequences inserted between the Ψ site and the *loxP* site will be incorporated into recombinant viruses in a Cre recombinase-catalyzed recombination between pAdlox and 95. (Bottom) A map of shuttle vectors for making gutless adenoviruses. pAB and ploxAB are identical except that ploxAB has a *loxP* site inserted at position 194 between the left ITR and the packaging site. The positions of *Clat* sites are marked.

create ploxAB. *Sfi*I-digested ploxAB plasmid DNA and *Clat*-digested AdB-gal viral DNA were introduced into 293 cells by using CaPO₄ transfection. 95 was isolated by plaque purification using standard techniques. AdB-gal is an Ad5 recombinant made from a pCMV Ad B plasmid containing a β -galactosidase gene and *dsb24* (27). The expression cassette and β -galactosidase gene replace E1 sequences up to nucleotide 3328, and a deletion of nucleotides 2892 to 30470 is in the E3 region.

Viral DNA analysis. Total low-molecular-weight DNA was prepared as described by Hirt (16). Packaged viral DNA was prepared by using spermine precipitation to remove the unpackaged nucleic acids as follows. At 48 h after infection, a 10-cm-diameter dish of infected cells was harvested by suspending the cells in their media and centrifuging them in a clinical centrifuge. The pellet was suspended in 400 μ l of 10 mM Tris (pH 9)-1 mM EDTA; then 400 μ l of 0.5 M EDTA and 40 μ l of 500 mM spermine-HCl, mixed, incubating the mixture for 10 min on ice, and centrifuging it in a microcentrifuge at full speed for 4 min at 4°C. The supernatant was digested with 4 μ l of RNase A (10 mg/ml) for 10 min at 37°C. Next, 60 μ l of 10% sodium dodecyl sulfate, 20 μ l of 0.5 M EDTA, and 40 μ l of pronase (50 mg/ml) were added, and the sample was incubated 1 h at 40°C. This sample was extracted once with phenol-chloroform and then precipitated with isopropanol. The DNA was dried and suspended in 25 μ l of 10 mM Tris (pH 8)-1 mM EDTA, and a 4- μ l aliquot was digested with the appropriate enzyme. A 1-kb DNA ladder (Life Technologies) was run on all agarose gels for size standards. DNA fragments were resolved by electrophoresis through 1% agarose gels run in Tris-acetate-EDTA, visualized with ethidium bromide, and photographed. Band intensities were determined from photographic negatives, using a laser densitometer (Molecular Dynamics). The relative molar yield of each left-end fragment shown in Fig. 2a was calculated by correcting for background fluorescence, scaling for yield by using the 2.4-kb AdB-gal fragment, and then adjusting for the number of base pairs in each fragment.

Cell staining and flow cytometry. To determine relative amounts of recombinant β -galactosidase-expressing virus, 1 ml of lysate was used to infect a 6-cm-diameter dish of 293 cells. At 20 to 24 h postinfection, the cells were removed with trypsin and aliquots were loaded with fluorescein-digalactoside (FDG) for

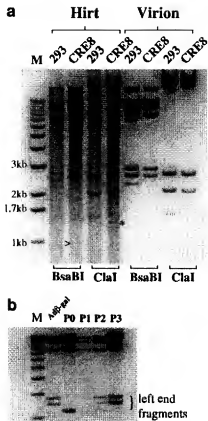


FIG. 2. (a) Restriction mapping of Ad β -gal and Ψ 5 DNA coinfecting into 293 or CRE8 cells. For each cell type, total low-molecular-weight (Hirt) DNA or packaged DNA was prepared and digested with BsaBI or ClaI and resolved by agarose gel electrophoresis. The viruses are identical except in their left ends. Digestion of Ad β -gal with BsaBI produces 2.4- and 2.8-kb fragments, and digestion with ClaI produces 2.1- and 2.5-kb bands in either cell type. ClaI cleaves between the *loxP* sites in Ψ 5, producing a 1.5-kb fragment from unrecombined Ψ 5 DNA. ClaI does not cleave the recombined form of Ψ 5 but does produce a 1.3-kb fragment from the excised circle marked with an asterisk. BsaBI cuts after the *loxP* sites and produces fragments of 2.2 kb from unrecombined Ψ 5 DNA and 0.98 kb (marked with >) from recombined Ψ 5 DNA. The lane M contains a 1-kb DNA ladder. (b) Negative selection against Ψ 5 virus in CRE8 cells. Lane M contains a 1-kb DNA ladder for size standards. The next lane is a digest of Ad β -gal DNA. Lane P0 is a reconstruction of 1% Ad β -gal and 99% Ψ 5 DNAs mixed and digested. Lanes P1, P2, and P3 are packaged DNAs prepared from three successive infections of mixed viruses containing 1% Ad β -gal and 99% Ψ 5 on CRE8 cells. All viral DNAs were digested with BsaBI, and the virus-specific left-end fragments are bracketed in the right margin.

60 s (22). Cells were stained with propidium iodide and analyzed with a Becton Dickinson FACScan. Data were collected from viable single cells.

Nucleotide sequence accession number. The GenBank accession number for pAdlox is U62024.

RESULTS

Selection against Ψ 5 virus. As noted above, the Ψ 5 virus is designed so that it can be selected against. When Ψ 5 is propagated in the presence of Cre recombinase, its *cis*-acting packaging sequence, which is flanked by *loxP* sites, is deleted, and the resulting genome cannot be encapsidated. To understand the selection process in detail, we examined Ψ 5 virus grown in nonselective (293) or selective (CRE8) cells. As a standard, we used a virus with a normal packaging sequence (Ad β -gal) in coinfections with Ψ 5. These two viruses are identical except for

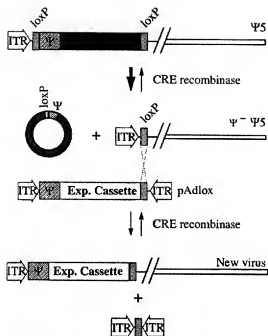


FIG. 3. Construction of an EI-substituted adenovirus by using Ψ 5 and a shuttle vector. Negative pressure on Ψ 5 is achieved by intramolecular recombination removing the packaging site in the first step. An intermolecular recombination between the shuttle vector and Ψ 5 then creates a new virus which has an intact packaging site and carries a recombinant gene, marked Exp. Cassette. The packaging site is labeled Ψ .

their left ends, which are easily distinguished by restriction analysis of the viral DNAs. Comparison of either total low-molecular-weight DNA (Hirt extraction) or packaged DNA provides the means to judge the relative packaging and replication efficiencies and to identify the recombination products.

Digestion of Ψ 5 DNA with BsaBI should produce a 2.2-kb fragment before recombination and a 0.98-kb fragment after recombination (BsaBI digestion of Ad β -gal produces 2.4- and 2.8-kb fragments). In the Hirt-extracted DNAs, the predicted 0.98-kb band appeared in CRE8 cells but not in 293 cells (Fig. 2a). The degree of recombination was over 90%, as judged by the relative intensity of the 2.2- and 0.98-kb bands. The amount of total Ψ 5 left ends in 293 or CRE8 cells relative to the amount of Ad β -gal left ends was constant. In the ClaI digest of Hirt DNAs, the 1.5-kb Ψ 5 left-end fragment was greatly reduced in CRE8 cells and there was a faint fragment of 1.3 kb, the predicted size of the excised circle (ClaI digestion of Ad β -gal yields 2- and 2.4-kb fragments).

If the packaging site was removed from Ψ 5 in CRE8 cells, then Ψ 5 DNA should be underrepresented in the packaged DNA from these cells. This was in fact the case. In 293 cells, the yields of the two DNAs were about the same. However, for growth in CRE8 cells, the Ψ 5 left-end fragment was about 10% of the Ad β -gal fragments when the DNA was digested with either enzyme. Of the Ψ 5 DNA which was packaged, the BsaBI digestion shows that it was unrecombined, since the 2.2-kb band was present and the 0.98-kb band was absent from the packaged DNA. These data show that Ψ 5 virus was subjected to a strong negative selection in CRE8 cells.

To confirm that the Ad β -gal virus rapidly overgrows the negatively selected Ψ 5 virus in CRE8 cells, we mixed it with Ψ 5

TABLE 1. Comparisons of Cre-lox- and homology-driven recombination and of viral and plasmid DNAs as sources of the virus backbone

Shuttle vector	Mode of recombination	Type of donor DNA		% lacZ-positive cells in FDG assay			
		Viral	Plasmid	3 days	7 days	10 days	14 days
pAdlox, cut	CRE-lox	Ψ5		1.7	100		
pAdCMV B, cut	Homology	Ψ5		0.2	100		
pAdCMV B	Homology		pBHG10	0	0	0.65	100
	Homology		pBHG10	0	0	0	0

at a ratio of 1 to 100 and infected CRE8 cells serially three times. At each passage, we prepared packaged DNA and monitored the ratios of the two viruses by restriction analysis. Figure 2b shows that in the course of three passages, the Ψ5-specific fragment all but disappeared and the Adβ-gal fragments became predominant.

E1-substituted virus construction. By using selection against Ψ5, it should be possible to make a recombinant adenovirus carrying nonviral DNA in place of the E1 genes by cotransfecting a shuttle vector with a loxP site (pAdlox) and Ψ5 DNA into a CRE8 cells (Fig. 3). In the first step of the reaction, Cre recombinase should catalyze recombination between the two loxP sites in Ψ5, removing the packaging site from the virus. In the second step, Cre recombinase should catalyze a recombination between Ψ5 and pAdlox, transferring the recombinant genes into Ψ5. The resulting recombinant virus will now have a single loxP site and therefore will have a considerable growth advantage over Ψ5 in CRE8 cells. This growth advantage should generate virus stocks comprised predominantly of the recombinant virus.

We compared our Cre-lox recombination technique to a popular overlap recombination method for production of recombinants (3). We set up a series of recombinations between transfected adenovirus donor DNAs and shuttle vectors and then prepared lysates 3, 7, 10, and 14 days after transfection and screened for the presence of lacZ-positive virus, using the FDG assay (Table 1; see Materials and Methods). These recombinations were used to examine the effects of two factors, the source of the donor DNA and the mechanism of recombination. First we transfected either Ψ5 viral DNA or pBHG10 (3) plasmid DNA as the donor genome along with shuttle vectors marked with lacZ genes. Second, we combined Ψ5 donor DNA with lacZ-marked shuttle vectors having either a loxP site for Cre-lox recombination or a 5.5-kb adenovirus fragment for overlap recombination. Recombination into Ψ5 viral DNA by either method produced a lacZ virus by 4 days, with Cre-lox recombination being slightly more efficient. In contrast, overlap recombination into pBHG10 plasmid DNA required 10 days before lacZ virus appeared, and then it did so only when the linear shuttle vector was used.

An important factor in the recombination process might be the topology of the shuttle plasmid. To test this, we transfected pAdlox plasmids carrying a β-galactosidase marker gene (pAdlox β-gal) either uncut or treated in various ways along with Ψ5 viral DNA (Fig. 4a). After 3 days, we prepared lysates and titrated them for β-galactosidase-positive virus by the FDG assay (Fig. 4b). All of the transfections produced recombinant virus but with a substantial variation in efficiency depending on the treatment of the plasmid. Cutting the pAdlox plasmid at both ITRs (SfiI) or at both ITRs plus cutting off the right ITR (SfiI plus ApaI) produced equally high yields of recombinant virus. Cutting the plasmid with ScaI such that the ITRs remained buried in plasmid sequences reduced the yield to 17% of SfiI-cut plasmid, and circular plasmid produced the least

amount of recombinant virus, at about 4% of the rate of plasmid cleaved at the ITRs.

Next we used transfection to introduce the shuttle plasmid and compared infection with transfection to introduce Ψ5 DNA. We transfected cells with the shuttle vector and infected them with Ψ5 virus at various times relative to the infection, and we cotransfected one sample as before (see Materials and Methods for details). We used the virus produced from each sample to infect CRE8 cells and performed a restriction analysis on the packaged DNA to monitor the ratios of the donor and recombinant viruses (Fig. 5). Cotransfection of Ψ5 and pAdlox β-gal produced a virtually donor-free stock of recom-

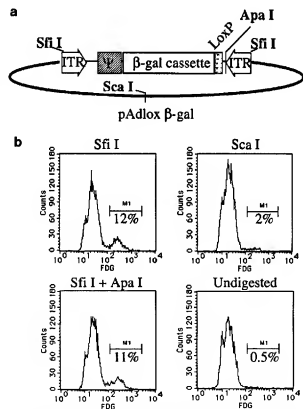


FIG. 4. (a) The pAdlox β-gal plasmid and the restriction enzyme cleavage sites used to assess the effect of shuttle vector topology on recombination. Cleavage with SfiI excises a fragment capable of replicating in adenovirus-infected cells. Cleavage with ApaI in addition to SfiI cuts off the right origin of replication, and cleavage with ScaI linearizes the plasmid but leaves the origins attached to bacterial DNA. (b) FDG analysis of recombination between Ψ5 and pAdlox cleaved in various ways. For quantitation, only the five single cells were used. The signal under the M1 bar is from the β-galactosidase-expressing cells.

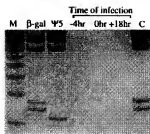


FIG. 5. Comparison of infection versus transfection as a means to introduce Ψ 5 DNA for recombination. The lanes marked β -gal and Ψ 5 are digests of $\text{Ad}\beta$ -gal and Ψ 5 DNAs to show the positions of the distinctive fragments. Lanes marked -4hr, 0hr, and 18hr are digests of viral DNA from infection of Ψ 5 virus at various times relative to transfection of pAdlox β -gal; the sample marked C resulted from cotransfection of Ψ 5 and pAdlox β -gal DNAs (see Materials and Methods for details). Packaged DNA from infection into CRE8 cells was digested with *Bsa*BI in all cases. The lane marked M is the 1-kb ladder DNA.

binant virus in 10 days. In contrast, infection of the donor virus combined with transfection of the shuttle plasmid produced less recombinant virus and a significant amount of donor contamination in a similar time frame.

In the foregoing experiment, there was very little donor virus DNA in cotransfected samples after the second passage through CRE8 cells. To determine more precisely how effectively the donor virus was removed during successive passages through CRE8 cells, we took the virus mixture from the cotransfection and passed it two more times through CRE8 cells, making lysates at each passage. Lysates which were stained with X-Gal and neutral red to distinguish the recombinant virus plaques from Ψ 5 or mutant viruses were then diluted and infected into 293 cells for plaque analyses (Table 2). The initial transfection (passage 1) contained 30% Ψ 5 virus. One passage through CRE8 cells reduced the Ψ 5 virus to 3%, in agreement with the restriction pattern seen in Fig. 5. A further passage through CRE8 cells reduced the concentration of donor virus to 0.2%.

TABLE 2. Reduction of Ψ 5 donor virus by passage through CRE8 cells

Passage no.	No. of blue plaques	No. of clear plaques	% Donor
P1	2×10^7	6×10^6	30
P2	1.5×10^8	5×10^6	3
P3	1×10^8	2×10^7	0.2

Gutless virus construction. Since the selection against Ψ 5 functions in *cis*, it should be a good helper virus. Therefore, we decided to use Ψ 5 to complement growth of an adenovirus vector which was missing all viral genes, a so-called gutless virus. Once again we used Ψ 5 virus, CRE8 cells, and a shuttle plasmid in an interacting fashion (Fig. 6). The shuttle plasmid carrying the gutless virus genome, pA β , was based on pCMV Ad (see Materials and Methods). It contained two ITRs with a packaging sequence in the left end, a β -galactosidase expression cassette as a marker, and a large piece of λ phage DNA as a nonfunctional substitute for the missing adenovirus DNA (Fig. 1b). In initial experiments, we determined the best scheme to turn pA β plasmid into a virus by examining two sets of options, using either virus or viral DNA as a source of Ψ 5 genomes and using either circular pA β or pA β cleaved at the ITRs as a source of the gutless genomes. The best combination was obtained by using cotransfection of the Ψ 5 DNA with *Sfi*I-digested pA β DNA, exactly the same conditions as we used for constructing E1-substituted viruses (data not shown).

Next, we tried to generate enough gutless virus for characterization. We cotransfected gutless plasmid with Ψ 5 viral DNA into CRE8 cells, overlaid the cells with agar, and stained them for β -galactosidase activity at 7 days. About 90% of the approximately 50 plaques per dish turned blue, indicating the probable presence of the A β gutless virus. We amplified the viruses from these plaques and restriction mapped their DNAs. All of the viruses that we amplified appeared to be derivatives of Ψ 5. A fragment that should have contained the left ITR, first *loxP* site, and packaging site of Ψ 5 comigrated

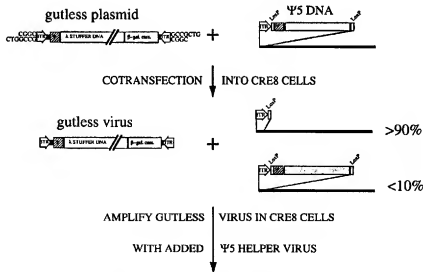


FIG. 6. Construction of a gutless adenovirus vector. In the first step, shuttle plasmid cleaved at the ITR is transfected into CRE8 cells with Ψ 5 DNA. The proteins from Ψ 5 convert the shuttle plasmid DNA to a molecule which is replicated by adenovirus DNA polymerase. The gutless virus is then encapsidated into adenovirus capsids. Several rounds of growth are necessary to amplify the gutless virus. At each round, more Ψ 5 virus is added to ensure that all cells contain the helper virus.

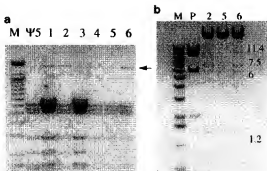


FIG. 7. (a) Restriction analysis of packaged gutless and $\Psi 5$ DNA from infected CRE8 cells. The DNA was digested with *Bgl*II. There are no *Bgl*II sites in the loxAB gutless virus. Lanes: M, 1-kb ladder plus 16.5- and 33.5-kb fragments; $\Psi 5$, $\Psi 5$ DNA; 1 to 6, isolates of loxAB plus $\Psi 5$. The arrow marks the position of loxAB DNA. (b) Restriction analysis of loxAB and $\Psi 5$ DNAs with *Cla*I. $\Psi 5$ DNA contains one site at base 1473. The predicted sizes of the loxAB fragments are 0.5, 1.2, 6, 7.5, and 11.4 kb. Lanes: M, 1-kb ladder; P, ploxAB cut with *Cla*I; 2, 5, and 6, isolates 2, 5, and 6 from panel a cut with *Cla*I. (Many of the bands from ploxAB do not match their cognate bands in loxAB, as the *Cla*I sites in the plasmid are methylated.)

instead with the equivalent fragment from pAB, while all of the rest of the fragments were consistent with $\Psi 5$ (data not shown). Thus, it appeared that pAB and $\Psi 5$ had recombined through homologous sequences somewhere between the two loxP sites in $\Psi 5$. Since there are no loxP sites in pAB, such a recombinant would have only one loxP site, giving the recombinant a strong advantage over $\Psi 5$ for growth in CRE8 cells. To prevent this, we added a loxP site to pAB between the left ITR and the packaging sequence, making ploxAB (Fig. 1). Any recombinant would then be subjected to the same selection as $\Psi 5$.

After cotransfecting *Sfi*I-digested ploxAB DNA and $\Psi 5$ viral DNA and staining for β -galactosidase activity as described above, we selected 12 blue plaques and amplified the loxAB gutless virus on CRE8 cells by successive passage of the virus onto 10^4 , 10^5 , 10^6 , and finally 10^7 cells. At each step, we added enough $\Psi 5$ virus (multiplicity of infection of 5) to ensure that all of the cells were infected by the helper virus. Restriction analysis of packaged DNA from CRE8 cells infected with a portion of the virus from the 10^7 cell lysates showed that 10 of 12 contained an appreciable amount of loxAB DNA (data for the first six are shown in Fig. 7a). The amount of loxAB DNA varied from a few percent to 20% of the total amount of virus. Two of the isolates were passaged further by infection into CRE8 cells without supplemental $\Psi 5$ virus. The amount of loxAB DNA never increased above 20% of the amount of the helper virus. The yield of loxAB virus was about 50 infectious particles per cell by the FDG assay.

To assess the integrity of the loxAB virus, we analyzed fragments produced from a *Cla*I digestion of several of the isolates (Fig. 7b). *Cla*I digestion produced five major fragments from loxAB, all of which migrated at their predicted sizes. We confirmed the loxAB virus would transduce β -galactosidase activity into 293 and CV1 cells (data not shown).

Isolates 1 and 3 shown in Fig. 7a contained a large amount of helper virus DNA, indicating that $\Psi 5$ had escaped selection. To determine the nature of the $\Psi 5$ virus after the transfection and amplification process, we plaque purified viruses from these samples, taking eight plaques for each isolate, from which we prepared DNA. Restriction analyses of these DNAs demonstrate that many contain deletions while some might be

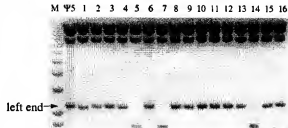


FIG. 8. Restriction analysis of $\Psi 5$ mutants with *Bst*BI. The position of the left-end fragment from $\Psi 5$ is marked. The DNA was from 16 plaque isolates purified on 293 cells. Lane M, size markers.

intact (Fig. 8). Taking representative viruses, we amplified two segments containing the loxP sites by PCR and then sequenced the PCR products. The left loxP sites were intact. However, in all cases, the right loxP site was missing (data not shown).

To further analyze the stability of $\Psi 5$ in CRE8 cells, we passaged $\Psi 5$ virus through CRE8 cells. After eight passages, the virus began to grow well. Once again, we plaque purified isolates from the passaged virus and subjected the DNA to restriction analyses and sequencing. Of 10 viruses, 1 was still $\Psi 5$ and the rest had deletions of one of the loxP sites (data not shown).

DISCUSSION

We have developed two methods for constructing recombinant adenoviruses by using Cre-lox recombination. Both methods rely on a cis-acting negative selection against an E1-deleted adenovirus that we call $\Psi 5$ (Fig. 1a). The selection operates through a pair of directly repeated loxP sites flanking the packaging site. Cre recombinase catalyzes recombination between these loxP sites, excising the packaging site (Fig. 3). Such a deletion renders the affected viral chromosome unusable for packaging but leaves all other viral functions intact (15). These properties make $\Psi 5$ an excellent helper virus for making recombinant adenoviruses.

Since high selection efficiency was critical for the success of our virus-building plans, we performed a detailed analysis of $\Psi 5$ growth (Fig. 2a). When $\Psi 5$ was grown in cells making Cre recombinase, over 90% of the molecules were recombined, and the yield of $\Psi 5$ virus was about 10% of the yield of the unselected $\Psi 5$ virus. The circular recombination product was present at a lower concentration than the deleted viral chromosome. The fact that the excised fragment was visible at all indicates that recombination was active during DNA replication. The relatively small quantity of the excised fragment may suggest that substantial recombination occurred before DNA replication. Replication of $\Psi 5$ DNA was normal under selective conditions. These results clearly show that lox sites, Cre recombinase, and recombination do not impair adenovirus replication, in agreement with previous findings (1). Further, the negative selection was apparently by a mechanism of preferential packaging resulting from the deletion of the packaging site.

We confirmed that the selection efficiency was sufficient to remove $\Psi 5$ from a mixture of two E1-substituted viruses (Fig.

2b and Table 2). Having two viruses in the same cell raises questions about the genetic stability of the selection, since adenoviruses recombine at a high rate. Recombination between $\Psi 5$ and a viral vector without flanking *loxP* sites could produce a version of $\Psi 5$ which would escape selection. Despite this, the negative selection was able to rapidly remove $\Psi 5$ from a mixture containing another E1-substituted adenovirus.

Apart from selection efficiency, the yield of recombinant vectors is controlled by the conversion rate of transfected DNA into replicating adenovirus DNA. Two sets of findings bear on this process. First, from highest to lowest, the transfection efficiencies for various adenovirus DNAs are the following: viral DNA with terminal protein attached, protease-treated viral DNA, plasmid DNA cleaved at the ends of the ITRs, and circular DNAs or DNAs cleaved distal to the ITRs (5, 14, 21, 26). Second, a plasmid-derived DNA bounded by ITRs will replicate to 10,000 copies per cell if cotransfected with viral DNA but will replicate poorly if the viral DNA is introduced by infection (14). Thus, the yield of viral vectors was highest when we transfected shuttle plasmids that were cleaved next to the ITRs (Fig. 4b and Table 1). Similarly, the yield was higher with protease-treated viral DNA than with pBH10 plasmid as the donor DNA (Table 1). Finally, cotransfection of shuttle plasmid with $\Psi 5$ DNA proved more efficient than transfection of shuttle DNA and infection with $\Psi 5$ virus at producing E1-substituted or gutless viruses (Fig. 5 and data not shown).

The final factor affecting the yield of E1-substituted viruses was the topology of the shuttle plasmid. We found that shuttle vector linearized at a site distal to the adenovirus sequences produced more recombinants than circular vector (Fig. 4b; compare uncut to *ScaI* cut). One explanation of these data is that the Cre-*lox* recombination reaction should be sensitive to the topology of the shuttle vector. The products of recombination between linear shuttle vector and $\Psi 5$ DNA are two molecules, each with one *loxP* site. The product of recombination between a circular shuttle vector and $\Psi 5$ DNA is a single molecule with two *loxP* sites. Chromosomes with two *loxP* sites are unstable, as they can rapidly recombine back to the two starting molecules by intramolecular recombination. Recombinants with single *loxP* sites are more stable, since their reversion occurs by much slower intermolecular recombination.

Applying these findings, we devised a plan to create E1-substituted viruses with high efficiency (Fig. 3). $\Psi 5$ serves as a donor virus contributing the viral backbone to the new virus. A shuttle plasmid (pAdlox) cleaved at the ITRs provides the recombinant gene(s) and a packaging site without flanking *loxP* sites. CRE8 cells supply Cre recombinase, which both recombines the shuttle and donor vectors and eliminates the $\Psi 5$ virus. As the new viruses produced by this arrangement have only one *loxP* site, they can rapidly overgrow $\Psi 5$ (Table 2).

There are now several improved methods for making E1-substituted recombinant adenoviruses. Two such methods are driven by negative selection schemes. One method also uses a selection in *cis*, based on a packaging site deletion (3). The second uses a selection in *trans* against herpes virus thymidine kinase to remove the donor virus (17). Both of these methods use homologous recombination to construct the recombinant viruses. Cre-*lox*-mediated recombination has also been used to construct herpesviruses in an *in vitro* recombination scheme based on negative selection in *trans* (9). Fundamentally different approaches using reconstruction of the entire virus by homologous recombination either as yeast artificial chromosomes, as plasmids in bacteria, or as a virus in 293 cells have also been used to create recombinant adenoviruses (5, 18, 21).

The chief advantages of using the technique described here is that it is simple and highly efficient (Table 1). The major

problem in using viral DNA as a source of donor DNA is having to purify the recombinants away from the donor virus. In this method, the purification was accomplished during amplification of the recombinant virus by a tight negative selection (Fig. 2b and Table 2). A secondary consideration in the choice of DNA for recombination is that plasmid DNA is much easier to prepare than viral DNA. While viral DNA is certainly more difficult to purify, it is also more stable, as the viral genome is under continuous selection during growth. In contrast, the viral sequences in plasmids are unselected. Finally, Cre-*lox* recombination enables the use of a small shuttle vector which is extremely easy to manipulate.

In our next method, we created an adenovirus vector which was missing all viral genes, or a gutless virus. The overall process was quite similar to one that we used for making E1-substituted viruses except that $\Psi 5$ served as a helper virus (Fig. 6). Because this method relies on a genetic selection to remove the helper virus, the whole packaging capacity of adenovirus can be used for recombinant DNA. Additionally, the biological separation can easily be adapted for large-scale production of virus for use in gene therapy. In contrast, two recent reports have detailed methods for producing gutless viruses based on CsCl density gradient separations to remove the helper virus, a process which is difficult and expensive to scale up (7, 19).

Fundamentally, there should be two factors which limit the purity of gutless virus produced with a *cis*-selected helper virus. First is the stability and efficiency of selection against the helper virus. Second is the ability of the gutless virus to be replicated and packaged by the helper virus. From our data, it appears that neither the helper nor the gutless virus performed optimally.

Homologous recombination appeared to be one source of instability. While recombination between $\Psi 5$ and a second virus was not a problem with E1-substituted viruses, such a recombinant did take over in the gutless virus case. We nullified the effects of recombination by placing a *loxP* site in the gutless virus before the packaging site, making ploxAB (Fig. 1). Any recombination in the packaging sequence between the two viruses now regenerates the initial molecules.

A second process that allowed $\Psi 5$ to escape selection was mutation of the *loxP* sites. We found that $\Psi 5$ virus mutated by deleting one of the two *loxP* sites. Since the Cre-*lox*-based selection need be applied only in the final growth of the gutless virus, this instability is not a problem. The simple solution is to perform initial growth of a gutless vector in 293 cells, amplifying the gutless virus by using a positive selection based on a marker gene in the gutless virus.

Low yield of the gutless virus adds to any problem with the stability of the helper virus. This may explain why the stability of $\Psi 5$ was not a problem in creating E1-substituted viruses but was a problem for making gutless viruses. The E1-substituted viruses have everything needed for viral growth in 293 cells. Even with $\Psi 5$, the yield of gutless virus from each cell was still substantially less than that of a wild-type virus. Our gutless virus has the minimal sequences needed for replication and packaging of an adenovirus DNA, similar to the plasmids used in two other studies (7, 19). In one of these studies, a dystrophin-bearing gutless virus similar in size to our virus also produced little virus per cell (19). The simplest interpretation of these data is that both gutless viruses are deficient in some function. The deficit could be in any function requiring a *cis*-active component, such as replication, packaging, removal of the viral core proteins, directing the viral DNA to a proper location, stabilizing the DNA in some fashion, or a structural role of DNA in viral particles.

A likely source of problems is in DNA packaging. There could easily be auxiliary sequences that increase packaging efficiency. Alternatively, since the lower limit for packaging has yet to be defined, it is possible that there is a strong effect of length on adenovirus packaging. This notion is especially credible. Both our lox Δ A virus and the dystrophin gutless virus are significantly smaller than normal, 75% or less of the size of the wild type. While these viruses were stable, a much smaller virus carrying only a lacZ cassette was extremely unstable, adding length by multimerizing or recombining with the helper (7). Thus, low yields and evidence of pressure to increase chromosomal length could indicate a strong preference for packaging full-length DNA. If this theory is correct, the low yield that we obtained could be easily corrected by inserting more stuffer DNA to increase the size of the gutless virus to 36 kb.

In summary, Cre-lox recombination is a powerful tool for genetic manipulation of adenoviruses. We present one application for constructing new E1-substituted adenoviruses which is an efficient alternative to the existing overlap recombination techniques. By this method, the viruses are almost pure without resorting to plaque purification. Thus, a working stock of virus can be produced for initial experiments in 10 days. In a second application, we demonstrate that a negatively selected helper virus can be used to support the growth of an adenovirus deleted of all viral coding sequences. This system, with some changes, should produce highly enriched gutless virus preparations in very large quantities for gene therapy.

ACKNOWLEDGMENTS

We thank Olivier Danos, Thomas Shenk, Richard Mulligan, Craig Jordan, Sean Sullivan, and John Forsayeth for critical reading of the manuscript or helpful discussions.

REFERENCES

- Antton, M., and F. L. Graham. 1995. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.* 69:4600-4606.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, New York, N.Y.
- Bett, A. J., W. Haddad, L. Preve, and F. L. Graham. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* 91:8802-8806.
- Bett, A. J., L. Preve, and F. L. Graham. 1995. Packaging capacity and stability of human adenovirus type 5 vector. *J. Virol.* 67:5911-5921.
- Chartier, C., E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, and M. Mehtali. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* 70:4805-4810.
- Chinnadurai, G., S. Chinnadurai, and J. Brucsa. 1979. Physical mapping of a large-plaque mutation of adenovirus type 2. *J. Virol.* 32:623-628.
- Fisher, K. J., H. Choi, J. Burda, S. Chen, and J. M. Wilson. 1996. Recombinant adenoviruses deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* 217:11-22.
- Dai, Y., E. M. Schwarz, D. Gu, W. Zhang, N. Sarvetnick, and I. M. Verma. 1995. Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc. Natl. Acad. Sci. USA* 92:1401-1405.
- Gager, P. J., B. Sauer, M. Levine, and J. C. Glorioso. 1992. A cell-free recombination system for site-specific integration of multicistronic shuttle plasmids into the herpes simplex virus type 1 genome. *J. Virol.* 66:5509-5515.
- Grable, M., and P. Hearing. 1990. Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.* 64:2047-2056.
- Grable, M., and P. Hearing. 1992. *cis* and *trans* requirements for the selective packaging of adenovirus type 5 DNA. *J. Virol.* 66:723-731.
- Graham, F. L., J. Smiley, W. C. Russell, and R. Narin. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-74.
- Graham, F. L., and A. J. van der Eb. 1972. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
- Haray, S. F., M. Lewandowski, G. Martin, and W. H. Hall. Unpublished data.
- Hay, R. T., N. D. Slow, and I. M. McDougall. 1994. Replication of adenovirus mini-chromosomes. *J. Mol. Biol.* 175:493-510.
- Hearing, P., R. J. Samulski, W. L. Wishart, and T. Shenk. 1987. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* 61:2555-2558.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
- Inler, J. L., C. Chartier, A. Dieterle, D. Dreyer, M. Mehtali, and A. Pavirani. 1995. An efficient procedure to select and recover recombinant adenovirus vectors. *Gene Ther.* 2:263-268.
- Kotzer, G., F. Spencer, S. Tugendreich, C. Connelly, and P. Hieter. 1994. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. *Proc. Natl. Acad. Sci. USA* 91:6186-6190.
- Kochanek, S., P. R. Clemens, K. Mitani, H. H. Chen, S. Chan, and C. T. Caskey. 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β -galactosidase. *Proc. Natl. Acad. Sci. USA* 93:5731-5736.
- McGrory, W. J., D. S. Baustista, and F. L. Graham. 1988. Short Communications: a simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. *Virology* 163:614-617.
- Miyake, S., M. Makimura, Y. Kasagawa, S. Harada, Y. Sato, K. Takamori, C. Tokuda, and I. Saito. 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing full-length virus genome. *Proc. Natl. Acad. Sci. USA* 93:1320-1324.
- Nolan, G. P., S. Fiering, J.-F. Nicolas, and L. A. Herzenberg. 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β -galactosidase activity after transduction of *Escherichia coli* lacZ. *Proc. Natl. Acad. Sci. USA* 85:2603-2607.
- Sauer, B. 1987. Functional expression of the Cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:2087-2096.
- Shenk, T. 1996. Adenoviridae: the viruses and their replication, p. 2111-2148. In B. N. Fields et al. (ed.), *Fields virology*. Raven Press, New York, N.Y.
- Slow, N. D. 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. *J. Virol.* 37:171-180.
- Tamanoi, F., and R. W. Stillman. 1982. Function of adenovirus terminal protein in the initiation of DNA replication. *Proc. Natl. Acad. Sci. USA* 79:2221-2225.
- Thimmappaya, B., C. Weinberger, R. J. Schaller, and T. Shenk. 1982. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. *Cell* 31:543-551.
- Yang, Y., Q. Li, H. C. J. Erft, and J. M. Wilson. 1995. Cellular and humoral immune responses to viral antigens create barriers to long-directed gene therapy with recombinant adenoviruses. *J. Virol.* 69:2004-2015.
- Young, C. S. II, T. Shenk, and H. S. Ginsberg. 1984. The genetic system, p. 125-172. In H. S. Ginsberg (ed.), *The adenoviruses*. Plenum Press, New York, N.Y.